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Whittington, Camilla M ; Griffith, Oliver W ; Qi, Weihong ; Thompson, Michael B ; Wilson, Anthony B

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# Seahorse Brood Pouch Transcriptome Reveals Common Genes Associated with Vertebrate Pregnancy

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## Abstract

**Viviparity (live birth) has evolved more than 150 times in vertebrates, and represents an excellent model system for studying the evolution of complex traits. There are at least 23 independent origins of viviparity in fishes, with syngnathid fishes (seahorses and pipefish) unique in exhibiting male pregnancy. Male seahorses and pipefish have evolved specialized brooding pouches that provide protection, gas exchange, osmoregulation, and limited nutrient provisioning to developing embryos. Pouch structures differ widely across the Syngnathidae, offering an ideal opportunity to study the evolution of reproductive complexity. However, the physiological and genetic changes facilitating male pregnancy are largely unknown. We used transcriptome profiling to examine pouch gene expression at successive gestational stages in a syngnathid with the most complex brood pouch morphology, the seahorse *Hippocampus abdominalis*. Using a unique time-calibrated RNA-seq data set including brood pouch at key stages of embryonic development, we identified transcriptional changes associated with brood pouch remodeling, nutrient and waste transport, gas exchange, osmoregulation, and immunological protection of developing embryos at conception, development and parturition. Key seahorse transcripts share homology with genes of reproductive function in pregnant mammals, reptiles, and other live-bearing fish, suggesting a common toolkit of genes regulating pregnancy in divergent evolutionary lineages.**

**Key words:** brood pouch, evolution, gestation, *Hippocampus abdominalis*, live birth, male pregnancy, reproduction, RNA-seq, Syngnathidae.

## Introduction

The evolution of novel traits is underpinned by genetic changes that encode new phenotypes. The genetic basis of phenotypic traits that are controlled by few genes is well known. For example, mosquitoes with a regulatory mutation in the gene encoding a single enzyme are insecticide resistant (Mitchell et al. 2012), and mutations in the Melanocortin 1 receptor gene influence coat color in animals ranging from mice (e.g., Hoekstra et al. 2006) to mammoths (Römler et al. 2006). In contrast, we know little about the genetic basis of complex traits that have resulted from changes in many genes, which have proven difficult to study using traditional genetic methods. Recent developments in deep sequencing technologies (RNA-seq) facilitate the examination of networks of genes responsible for the evolution of complex phenotypes. Viviparity (live birth) is one such trait, a highly developed form of reproduction requiring the evolution of a diverse set of adaptations involved in regulating the internal reproductive environment and provisioning developing embryos inside the parent. Viviparity has evolved from oviparity (egg laying) independently at least 150 times, across nearly

every major vertebrate lineage, including at least 23 times in fishes (Blackburn 2015). The repeated origin of viviparity by convergent evolution offers the opportunity to examine independent evolutionary origins of reproductive complexity and to uncover the processes by which viviparity evolves at the genetic level.

Syngnathid fishes (seahorses, pipefishes, and seadragons), a family of approximately 300 species, represent the only known examples of male pregnant animals (Stolting and Wilson 2007). The syngnathid brood pouch, derived from abdominal epithelium, is functionally equivalent to the amniote (mammal and reptile) uterus. Females transfer large, yolk-rich eggs into the pouch, which the male immediately fertilizes, assuring their paternity (Van Look et al. 2007). The complexity of the syngnathid brood pouch varies across the family (ranging from simple egg attachment sites through to the fleshy, fully enclosed brood pouch of seahorses), representing an excellent system in which to explore the evolution of reproductive complexity (Wilson et al. 2003; Wilson and Orr 2011). However, despite the important role of this group as an evolutionary model of sexual selection (Rosenqvist and

Berglund 2011), and widespread interest in the evolution of viviparity (Blackburn 2015), the genetic, physiological and endocrinological regulation of this unique form of reproduction remain poorly understood (Stolting and Wilson 2007; Scobell and MacKenzie 2011).

During gestation, the brood pouch undergoes significant morphological changes, including thickening, vascularization, development of epithelial microridges, and restructuring, as embryos embed into the pouch epithelium (Carcupino et al. 2002, 1997; Laksanawimol et al. 2006), morphological changes that parallel the restructuring of mammalian and reptilian uteri during pregnancy (Renfree 2010; Murphy and Thompson 2011). Although syngnathids lack the complex interdigitated placental structure found in mammals and some reptiles (Oconer and Herrera 2003), there is evidence of a pseudoplacenta in some species (Stolting and Wilson 2007), and the pregnant seahorse brood pouch epithelium conforms to the widely accepted definition of a placenta as an “apposition or fusion of fetal membranes to the uterine mucosa for physiological exchange” (Mossman 1937), with the pouch epithelium being functionally equivalent to the mucosal lining of the uterus. Brood pouch physiology differs among syngnathids, and pouch ultrastructure and function vary even among pouches of closely related species (Carcupino et al. 2002; Ripley and Foran 2006), suggesting that pouch function can evolve rapidly following divergence. However, some pouch activities appear to be conserved across taxa, including mechanical protection from predation and immunoprotection (Melamed et al. 2005; Roth et al. 2012). The brood pouch also functions in gas exchange and waste removal, as well as osmoregulation (Linton and Soloff 1964; Quast and Howe 1980; Azzarello 1991; Watanabe et al. 1999; Ripley 2009).

A major innovation in the evolution of viviparity is matrotrophy, the ability of mothers to provide supplementary nutrition to embryos during development. Evidence to support a patrotrophic function (i.e., supplementary nutrition from the father) of the syngnathid brood pouch is mixed, and pouch complexity does not necessarily reflect the degree of nutrient provisioning (Haresign and Shumway 1981; Ahnesjö 1992; Ahnesjö 1996; Ripley and Foran 2006, 2009; Kvarnemo et al. 2011). In particular, evidence for nutrient transport in seahorses is equivocal (Azzarello 1991; Boisseau 1967), and low rates of seahorse embryo survival in vitro suggest that supplementary nutrient provisioning from the father may not be essential (Linton and Soloff 1964).

The lack of data on brood pouch function in syngnathids limits our ability to identify how male pregnancy has evolved and to test for adaptive convergence in viviparity across vertebrates. Genomic techniques provide a unique opportunity to determine the genetic basis of complex traits, and have proven pivotal in studying female pregnancy in other viviparous animals (e.g., skink uterus, Brandley et al. 2012; elephant placenta, Hou et al. 2012; human placenta, Kim et al. 2012; Saben et al. 2014; livebearing *Poeciliopsis* fishes, Panhuis et al. 2011; porcine endometrium, Samborski et al. 2013). Previous studies have identified novel transcripts in the syngnathid brood pouch (Melamed et al. 2005; Harlin-Cognato et al.

2006; Small et al. 2013), but these studies have involved the pooling of tissue between multiple individuals and reproductive stages, limiting the potential for associating transcriptional changes with specific morphological restructuring and physiological processes occurring during gestation.

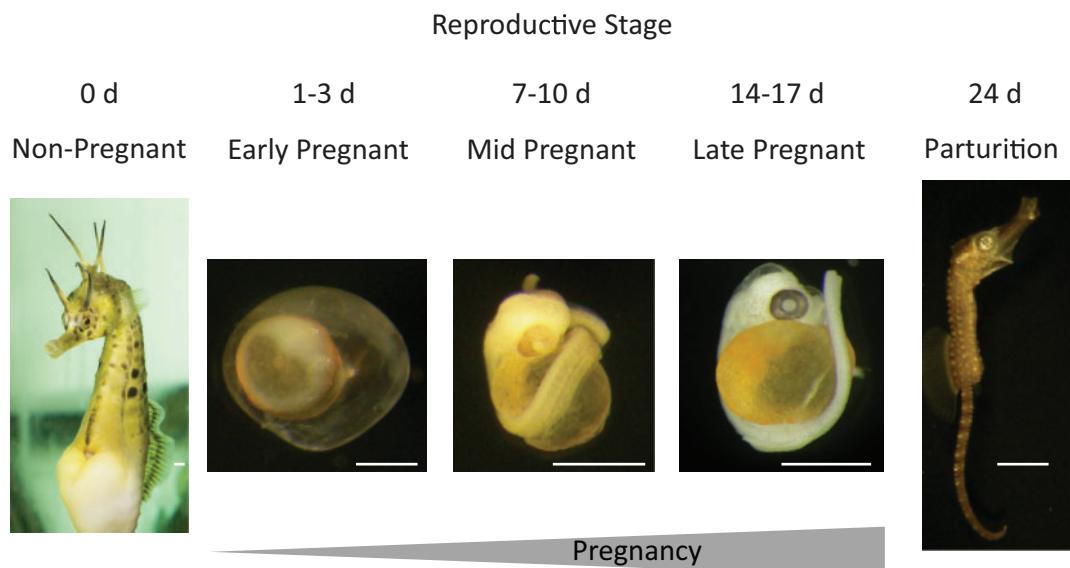
Here we use deep sequencing technologies to examine changes in pouch gene expression during pregnancy in the seahorse (*Hippocampus abdominalis*), a species exhibiting the most complex form of male pregnancy. Using an RNA-seq analysis of gene expression across multiple stages of pregnancy, we identify transcribed genes associated with crucial biological processes in the pregnant pouch through key stages of conception, development, and parturition. Although previous studies have identified gene expression differences during pregnancy using microarrays (Knox and Baker 2008), this is the first time that next-generation sequencing has been used across the full range of pregnancy in any animal, providing a uniquely fine-scale view of gene expression changes associated with reproductive function. We identify a diverse range of transcripts differentially expressed across pregnancy that are associated with known physiological functions of the seahorse brood pouch, a complex and uniquely derived reproductive organ.

The evolution of live birth requires a complex set of adaptations associated with embryonic development in a closed environment, including gas exchange, waste removal/storage, and nutrient transport to the developing embryo. The differential expression of genes associated with such functions may be facilitated through a number of mechanisms, including the pre-existing expression of transcripts in homologous tissues of a nonviviparous ancestor, the recruitment of genes into new expression domains in derived reproductive tissues, and/or the evolution of novel reproductive genes through mutation and/or gene duplication (True and Carroll 2002). We aimed to identify whether common genes are associated with pregnancy in seahorses and other vertebrates. To this end, we compare differentially expressed genes associated with reproductive function in the seahorse brood pouch with genes differentially expressed across pregnancy in other taxa to discover whether independent origins of viviparity are associated with a common set of genetic pathways.

## Results and Discussion

### Transcriptome Annotation and Analysis

Our transcriptomic analysis revealed the differential expression of genes at key stages of male pregnancy from conception to parturition, offering an unprecedented fine-scale view of gene expression changes across male pregnancy, a unique form of reproduction (fig. 1). Our transcriptome screen recovered 104–125 million paired end reads per pregnancy stage, which were assembled into 259,151 contigs (transcripts), approximately 35% of which were annotated to 16,688 unique Ensembl IDs on the basis of similarity to known genes in zebrafish and stickleback. In total, 3,158 transcripts were found to be differentially expressed between reproductive stages, a large number of which were differentially expressed between nonpregnant and pregnant animals (527,



**Fig. 1.** Stages of pregnancy for *Hippocampus abdominalis* pouch tissues sampled for the transcriptome ( $n = 3$  individuals per stage). A nonpregnant adult male seahorse is shown for the nonpregnant stage; note that pregnant males are virtually indistinguishable (Whittington et al. 2013). Thus, we represent later stages of pregnancy with photographs of the embryonic animals rather than the pregnant father. Early, mid, and late pregnancy photographs are representative of embryonic development at each stage (n.b. early pregnant and mid-pregnant embryos have been dechorionated), and the postparturition stage is represented by a newly released neonate. Scale bars = 0.5 mm. Embryo and neonate photographs provided by Stefan Sommer.

966, and 535 transcripts differentially expressed in nonpregnant animals compared with early-, mid-, and late-pregnancy, respectively; [fig. 2](#)).

#### Cluster Analysis

We carried out cluster analysis to partition the 3,158 differentially regulated transcripts into groups with consistent expression profiles. This analysis generated nine clusters including 21% of differentially expressed transcripts, and identified transcripts uniquely upregulated at individual time points (Clusters 1–5), transcripts consistently downregulated throughout pregnancy (Clusters 6 and 7) and transcripts showing consistently elevated expression profiles through successive stages of pregnancy (i.e., early-mid pregnancy [Cluster 8], mid-late pregnancy [Cluster 9]) ([fig. 3](#) and [supplementary table S1, Supplementary Material online](#)). This analysis identified a “parturition cluster” of 143 transcripts that are upregulated only after parturition, 78% of which could be annotated (Cluster 5, [fig. 3](#)). The putative functions of transcripts in these clusters are explored in our Gene Ontology (GO) analysis below (see also [supplementary table S2, Supplementary Material online](#)).

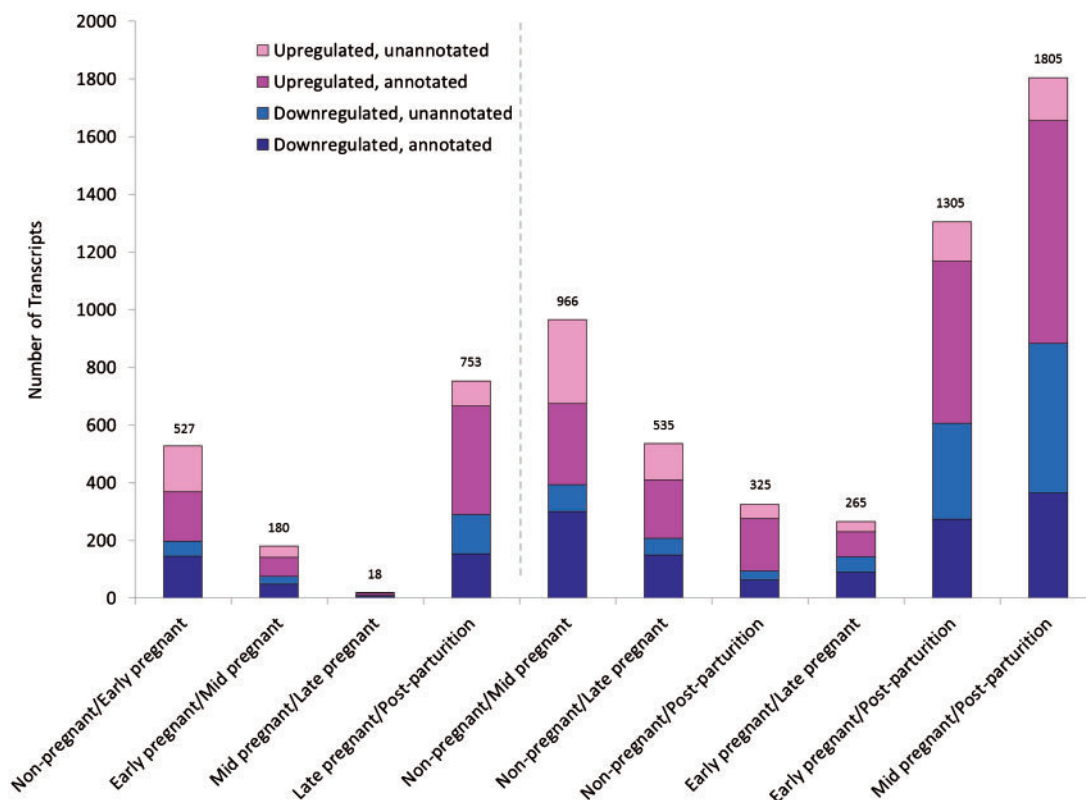
#### GO Annotation, Functional Grouping, and Pathway Analysis

GO annotation identifies broad functional categories of expressed genes from large gene expression data sets, and is particularly useful for evaluating systems-level changes in nonmodel organisms. GO annotation and functional grouping of differentially regulated annotated transcripts (2,030/3,158 annotated [64.3%]) (Huang et al. 2009) were used to identify the overrepresentation of specific functional groups in the male brood pouch at each reproductive stage. GO functional annotation of transcripts upregulated in pregnant

(early-, mid-, and late-pregnant) compared with nonpregnant seahorses identified 156 GO terms (ten of which were significantly enriched [ $P < 0.05$  before Benjamini–Hochberg correction]), including gene groups involved in transport, homeostasis, cytoskeleton organization, regulation of apoptosis, biosynthesis, immune regulation, tissue remodeling, development, metabolism, transcription, and phosphate-related processes ([supplementary table S3, Supplementary Material online](#)). GO functional annotation of transcripts downregulated during pregnancy revealed transcripts associated with 99 GO terms, 15 of which were significantly enriched. Downregulated GO functional groupings during pregnancy included kinase activity, metabolism, transcription, cellular organization, tissue remodeling, development, immune regulation, phosphate-related processes, and transport ([supplementary table S4, Supplementary Material online](#)).

Parturition was associated with some of the most dramatic changes in transcriptome activity, with greater than 750 transcripts exhibiting significant changes in activity after embryo release compared with the late-pregnant pouch ([fig. 2](#)). Postparturition animals showed the upregulation of 376 annotated transcripts associated with 104 GO terms, 10 of which were significantly enriched ([supplementary table S5, Supplementary Material online](#)). Upregulated transcripts after parturition included those implicated in phosphate-related processes, proteolysis, biosynthesis and metabolism, cellular organization, regulation of apoptosis, immune regulation, homeostasis, transcription, development, transport, and repair ([supplementary table S5, Supplementary Material online](#)). The number of annotated transcripts downregulated after parturition was lower (154; [fig. 2](#)), and downregulated transcripts fell into 17 GO





**Fig. 2.** Number of differentially expressed transcripts in pairwise comparisons (x axis) of pregnancy stages. Up/downregulation refers to the second reproductive stage for each comparison. The dashed line separates comparisons across sequential stages of pregnancy (left hand side) from all other pairwise comparisons (right hand side).

categories, only 1 of which was significantly enriched (supplementary table S6, Supplementary Material online). Functional groups included transcripts implicated in phosphate-related processes, protein transport, and transcription (supplementary table S6, Supplementary Material online). Although no GO terms remained significant after Benjamini–Hochberg correction for multiple comparisons, false-discovery rate corrections on large transcriptome data sets increase type II error rates, and may fail to detect true positives (Verhoeven et al. 2005). As such, we follow the recommendations of Huang et al. (2009) and report all functionally grouped GO terms as indicators of the overrepresentation of particular functional classes in our expression data. GO annotation using WebGestalt recovered comparable GO terms (supplementary tables S7–S10, Supplementary Material online).

Thirteen KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways were identified from the GO functional classes identified in the above comparisons (supplementary table S11, Supplementary Material online), including the mitogen-activated protein kinase (MAPK) pathway, which was upregulated in postparturition animals (see below)—none of these pathways remained significantly enriched after Benjamini–Hochberg correction. Similar results were obtained using WebGestalt, although a higher number of pathways (47) were detected to be significantly enriched, all of which survived Benjamini–

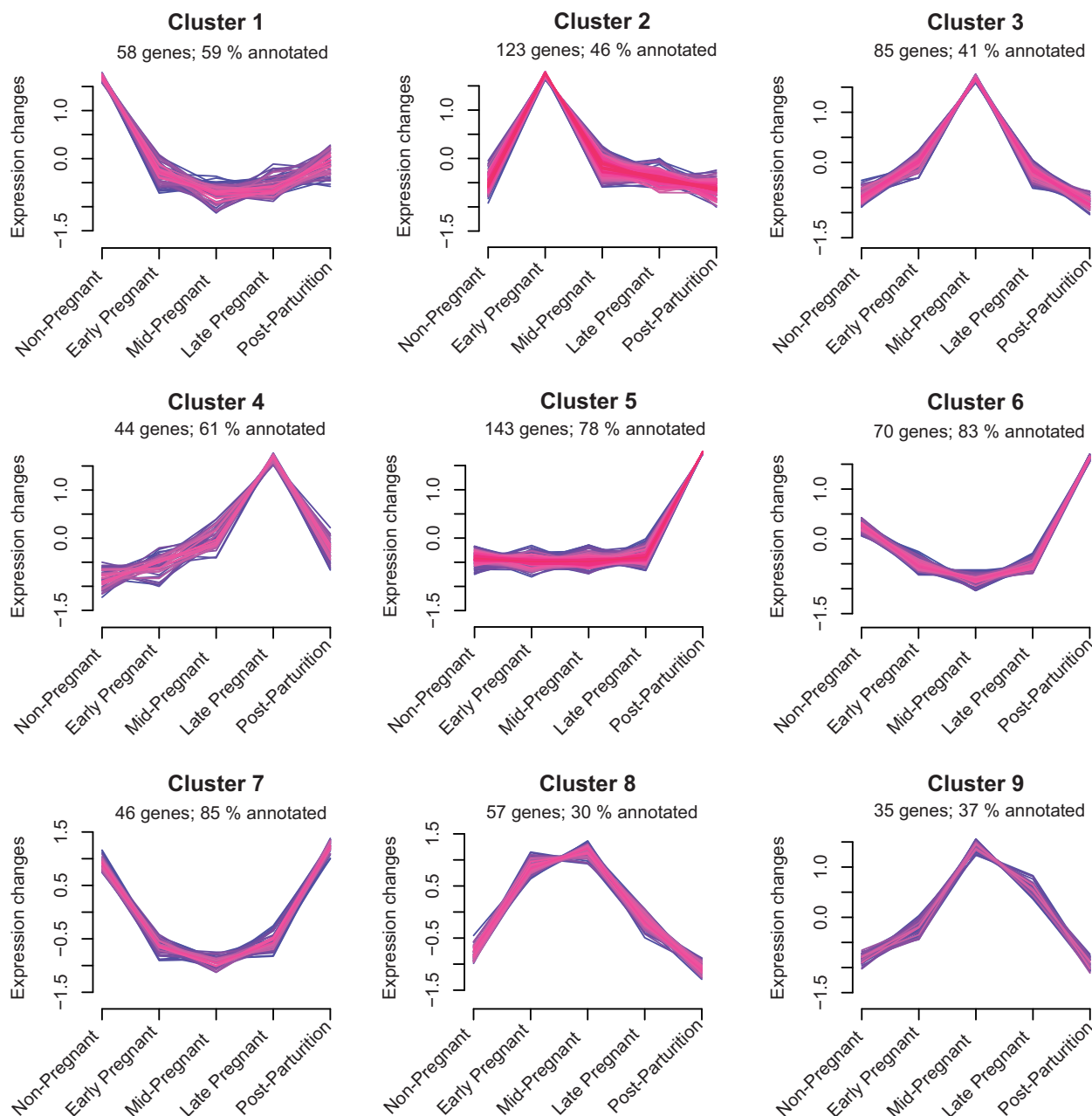
Hochberg correction (supplementary table S11, Supplementary Material online).

### Genes with Putative Function in Reproduction

In the following section, we highlight GO-annotated groups and differentially regulated transcripts known to be associated with reproductive function in other systems, including the top 30 differentially regulated transcripts by Benjamini-adjusted *P* value in each comparison, which were classified as highly differentially regulated (tables 1–3 and supplementary table S12, Supplementary Material online). The full expression profiles of the transcripts discussed here are provided in supplementary table S12, Supplementary Material online.

#### Tissue Remodeling: Restructuring of the Brood Pouch

After eggs are deposited into the seahorse brood pouch and fertilized, the pouch matrix undergoes marked restructuring (Stolting and Wilson 2007). Our expression analysis reveals a significant increase in the expression of genes involved in tissue remodeling during gestation, including several of the most highly upregulated transcripts across pregnancy (table 1 and supplementary table S12, Supplementary Material online). These genes function to regulate cell growth, proliferation, and death, and include mediators of focal adhesion and tissue remodeling in mammalian pregnancy (e.g., *SERPINE2*, Chern et al. 2011; *CAV1*, Turi et al. 2001). Apoptotic genes such as *GRIN1* and *PRKCD* are some of



**Fig. 3.** Cluster analysis of differentially expressed genes across pregnancy, partitioned into nine clusters. Expression changes (y axis) reflect standardized expression values, adjusted such that the mean expression per transcript is 0 and standard deviation is 1.

the most downregulated throughout seahorse pregnancy (table 2). Genes functioning in vasculogenesis (MCAM, ACVRL1, and EGFL7) are also upregulated during seahorse pregnancy (table 1), and may contribute to the maintenance of the highly vascularized pouch epidermis, which is thought to facilitate gas exchange and waste removal, an important prerequisite for the enclosed incubation of embryos in a thick layer of epithelial tissue (Laksanawimol et al. 2006).

#### Transport I: Nutrient Transporters and Patrotrophy

Genes associated with nutrient transport are expressed in the brood pouch of pregnant seahorses, highlighting potential candidate genes associated with paternal provisioning during pregnancy. As seahorse embryos have

been shown to survive in vitro incubation (Linton and Soloff 1964), paternal nutrient transport appears not to be essential, but embryos developing outside the parent experience developmental costs and reduced survival (Azzarello 1991). Our discovery of significantly upregulated nutrient transporter genes in the pregnant brood pouch is consistent with the hypothesis that the seahorse provides supplementary nutrition to embryos. Seahorse patrotrophy may be provided in the form of histotrophe (uterine milk), as occurs in some viviparous sharks and rays (Wourms 1981), which could act to supplement yolk lipids and inorganic ions provided by the mother, and help to digest maternally derived nutrients for embryonic uptake (Boisseau 1967).

**Table 1.** Significantly Upregulated Genes in the Pregnant Seahorse Brood Pouch (early-, mid-, late-pregnant) Compared with the Nonpregnant Pouch.

Gene Symbol	Gene Name	Putative Pouch Function	Cluster
<b>Tissue remodeling</b>			
— <sup>a</sup>	Si:ch211-195b13.1	Regulation of cell proliferation and apoptosis	—
A2M <sup>a</sup>	Alpha-2-macroglobulin	Protease inhibitor; innate immunity	2
ACVRL1	Activin A receptor type II-like 1	Blood vessel development	—
ARHGEF11	Rho Guanine Nucleotide Exchange Factor (GEF) 11	Decreases focal adhesion	—
CAV1	Caveolin 1	Focal adhesion	—
CLDN1	Claudin k	Tight junctions	—
DLX3	Distal-less homeobox 3	Tissue remodeling	2
EGFL7	EGF-like-domain, multiple 7	Vasculogenesis	—
KIAA1199 <sup>a</sup>	Cell Migration Inducing Protein, Hyaluronan Binding	Cell migration	—
MCAM	Melanoma cell adhesion molecule	Blood vessel development; cell adhesion	—
MMP19	Matrix metalloproteinase 19	Tissue remodeling	—
PHLDA1	Pleckstrin homology-like domain, family A, member 2	Regulation of cell growth	—
PPP1R12A	Protein phosphatase 1, regulatory (inhibitor) subunit 12A	Focal adhesion	—
PRSS59.1	Protease, serine, 59, tandem duplicate 1	Serine protease	—
SERPINE2 <sup>a</sup>	Serpin Peptidase Inhibitor, Clade E (Nexin, Plasminogen Activator Inhibitor Type 1), Member 2	Proteolysis; serine protease inhibition	—
SNAI1	Snail Family Zinc Finger 1	Regulates cell adhesion and movement	2
SVIL	Supervillin	Disassembly of focal adhesions	9
TMPRSS2	Transmembrane Protease, Serine 2	Tissue remodeling	3
<b>Gene expression</b>			
CALCOCO1 <sup>a</sup>	Calcium Binding And Coiled-Coil Domain 1	Transcriptional activator	8
JUNB	Jun B proto-oncogene	Transcription factor	—
NR4A3 <sup>a</sup>	Nuclear Receptor Subfamily 4, Group A, Member 3	Transcriptional activator	2
PUS10 <sup>a</sup>	Pseudouridylate Synthase 10	RNA processing	—
<b>Transport</b>			
ABCA12 <sup>a</sup>	ATP-binding cassette subfamily A member 12	Lipid transport	—
APOA1 <sup>a</sup>	Apolipoprotein A1	Lipid transport	—
APOA2	Apolipoprotein A2	Lipid transport	4
APOLD1	Similar to Apolipoprotein L	Lipid transport	—
APOM <sup>a</sup>	Apolipoprotein M	Lipid transport	9
AQP1	Aquaporin 1 (Colton Blood Group)	Water transport	4
AQP3	Aquaporin 3	Water transport	—
ATP10D	ATPase, Class V, Type 10D	Phospholipid transport	—
ATP2C1	ATPase, Ca <sup>++</sup> Transporting, Type 2C, Member 1	Calcium ion transport	9
ATP5L2	ATP Synthase, H <sup>+</sup> Transporting, Mitochondrial Fo Complex, Subunit G2	H <sup>+</sup> transport	—
CA2	Carbonic Anhydrase II	CO <sub>2</sub> transport; ammonia transport	—
CA4	Carbonic Anhydrase IV	CO <sub>2</sub> transport; ammonia transport	—
CETP	Cholesteryl ester transfer protein, plasma	Lipid transport	—
FABP1	Fatty Acid Binding Protein 1, Liver	Lipid transport	—
FABP3	Fatty Acid Binding Protein 3, Muscle And Heart (Mammary-Derived Growth Inhibitor)	Long chain fatty acid transport	—
FABP7	Fatty Acid Binding Protein 7, Brain1	Fatty acid transport	—
FTH1	Ferritin, heavy polypeptide 1	Iron ion transport	—
HSPA5 <sup>a</sup>	Heat Shock 70 kDa Protein 5 (Glucose-Regulated Protein, 78 kDa)	Protein transport monitoring	2
KCNK5 <sup>a</sup>	Potassium channel, subfamily K, member 5	Potassium ion transport	3
RHAG	Rhesus blood group-associated glycoprotein	CO <sub>2</sub> transport; ammonia transport	—
SEC23B <sup>a</sup>	Sec23 Homolog B ( <i>S. cerevisiae</i> )	Vesicle transport	—
SLC17A3	Solute Carrier Family 17 (Organic Anion Transporter), Member 3	Anion transport	3
SLC25A14	Solute carrier family 25 (mitochondrial carrier, brain), member 14	Mitochondrial transport	—
SLC25A22	Solute Carrier Family 25 (Mitochondrial Carrier: Glutamate), Member 22	Glutamate transport	—
SLC25A38	Solute carrier family 25, member 38	Mitochondrial transport	—

(continued)

Table 1. Continued

Gene Symbol	Gene Name	Putative Pouch Function	Cluster
SLC30A7	Solute Carrier Family 30 (Zinc Transporter), Member 7	Zinc ion transport	—
SLC31A2	Solute carrier family 31 (copper transporter), member 2	Copper ion transport	—
SLC35A1	Solute Carrier Family 35 (CMP-Sialic Acid Transporter), Member A1	Nucleotide sugar transport	—
SLC35B1 <sup>a</sup>	Solute Carrier Family 35 Member B1	Nucleotide sugar transport	2
SLC35D1	Solute Carrier Family 35 (UDP-GlcA/UDP-GalNAc Transporter), Member D1	Nucleotide sugar transport	—
SLC38A10	Solute Carrier Family 38, Member 10	Amino acid transport	3
SLC38A5	Solute Carrier Family 38, Member 5	Amino acid transport	—
SLC39A6	Solute Carrier Family 39 (Metal Ion Transporter), Member 6	Zinc ion transport	—
SLC41A1 <sup>a</sup>	Solute Carrier Family 41 (Magnesium Transporter), Member 1	Magnesium ion transport	—
SLC4A1	Solute Carrier Family 4 (Anion Exchanger), Member 1 (Diego Blood Group)	Chloride/Bicarbonate exchange (CO <sub>2</sub> transport)	4
SLC4A10	Solute Carrier Family 4, Sodium Bicarbonate Transporter, Member 10	Sodium/bicarbonate cotransport	2
SLC4A10	Solute Carrier Family 4, Sodium Bicarbonate Transporter, Member 10	Sodium/bicarbonate cotransport	—
SLC6A11A	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 12	GABA (neurotransmitter) transport	2
SLC6A11A	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 12	GABA (neurotransmitter) transport	—
TF <sup>a</sup>	Transferrin- $\alpha$ ; Rho-class glutathione S-transferase	Iron ion transport	—
Immune regulation			
CXCL12	Chemokine (C-X-C Motif) Ligand 12	Immune cell chemotaxis	—
F2RL	Coagulation Factor II (Thrombin) Receptor-Like 1	Immune response	—
F3	Factor 3-like	Immune response	—
HAMP <sup>a</sup>	Hepcidin antimicrobial peptide	Innate immunity (antifungal)	4
LCK	Lymphocyte-Specific Protein Tyrosine Kinase	T-cell selection and maturation	—
LECT2 <sup>a</sup>	Leukocyte cell-derived chemotaxin 2	Innate immunity (antibacterial)	—
TLR18 <sup>a</sup>	Toll-like receptor 18	Innate immunity (pathogen recognition)	9
Other			
— <sup>a</sup>	Ras-dva small GTPase	Nucleotide binding	—
— <sup>a</sup>	Uncharacterized protein		9
— <sup>a</sup>	Uncharacterized protein		4
ALDOC	Aldolase C, Fructose-Bisphosphate	Glycolysis	—
ASL	Argininosuccinate lyase	Ammonia detoxification	—
ASTL <sup>a</sup>	Astacin-like metallo-endopeptidase (M12 Family) (Patristacin)	Choriolysis	—
CALR	Calreticulin	Calcium binding and homeostasis	2
CALR3	Calreticulin 3	Calcium binding	2
CALU	Calumenin	Calcium binding	2
EDN2	Endothelin 1	Vasoconstrictor, vascular maintenance	4
EVA1C <sup>a</sup>	Eva-1 Homolog C ( <i>C. Elegans</i> )	Carbohydrate binding	9
EVA1C <sup>a</sup>	Eva-1 Homolog C ( <i>C. Elegans</i> )	Carbohydrate binding	3
FARSA <sup>a</sup>	Phenylalanyl-TRNA Synthetase, Alpha Subunit	Protein binding	2
HPX	Phosphoglucomutase 2	Carbohydrate metabolism	—
HPX <sup>a</sup>	Hemopexin	Metal ion binding	—
OVGP1 <sup>a</sup>	Oviductal glycoprotein 1, 120 kDa	Regulation of adhesion; embryonic protection	8
PKLR	Pyruvate kinase, liver and RBC	Glycolysis	2
S100A1	S100 calcium binding protein A1	Calcium and zinc binding	—
SELENBP1 <sup>a</sup>	Selenium Binding Protein 1	Selenium binding	—
TACR2	Tachykinin receptor 2	Reproductive function (smooth muscle)	—
ZNF365 <sup>a</sup>	Zinc finger protein 365	Nitrogen excretion	2

NOTE.— Cluster membership refers to the expression clusters shown in figure 3.

<sup>a</sup>Highly upregulated genes (the 30 most significantly upregulated genes in each pairwise comparison).

Our results indicate that genes involved in lipid transport are highly upregulated during seahorse pregnancy (table 1 and supplementary table S3, Supplementary Material online). During mammalian and some lizard pregnancies, free fatty acids cross the placenta for use by the fetus for

growth and development (Thompson et al. 1999; Duttaroy 2009; Griffith et al. 2013). In seahorses, the most significantly upregulated pouch transcript in all three stages of pregnancy compared with the nonpregnant pouch is Apolipoprotein M (APOM) (fig. 3: Cluster 9), a membrane-bound lipid



**Table 2.** Significantly Downregulated Genes in the Pregnant Seahorse Pouch (early-, mid-, late-pregnant) Compared with the Nonpregnant Pouch.

Gene Symbol	Gene Name	Putative Pouch Function	Cluster
<b>Cell growth, differentiation, and apoptosis</b>			
— <sup>a</sup>	Novel protein containing a C-type lectin domain	Regulation of cell growth	—
— <sup>a</sup>	Novel protein containing C-type lectin domains	Regulation of cell growth	—
— <sup>a</sup>	zgc:100868	Proteolysis	—
AXIN1	Axin 1	Protein kinase activity	—
CAD	Carbamoyl-Phosphate Synthetase 2, Aspartate Transcarbamylase, And Dihydroorotase	Regulation of cell growth	—
CAMK2N2 <sup>a</sup>	Calcium/Calmodulin-Dependent Protein Kinase II Inhibitor 2	Regulation of cell growth	—
CASP3	Caspase 3, Apoptosis-Related Cysteine Peptidase	Apoptosis	7
COL5A2 <sup>a</sup>	Collagen, Type V, Alpha 2	Extracellular matrix organization	7
CRISPLD2 <sup>a</sup>	Cysteine-Rich Secretory Protein LCCL Domain Containing 2	Regulation of extracellular matrix	1
DAG1	Dystroglycan 1 (Dystrophin-Associated Glycoprotein 1)	Basement membrane assembly; cell migration	—
DCN	Decorin	Regulation of extracellular matrix	—
DLX3	Distal-less homeobox 3	Tissue remodeling	7
FGFRL1	Fibroblast Growth Factor Receptor 1	Cell growth and differentiation	—
FIBIN <sup>a</sup>	Fin Bud Initiation Factor Homolog (Zebrafish)	Morphogenesis	—
FLOT2	Flotillin 2	Cell adhesion	—
GRIN1 <sup>a</sup>	Glutamate Receptor, Ionotropic, N-Methyl D-Aspartate-Associated Protein 1 (Glutamate Binding)	Apoptosis	—
HTRA1	HtrA Serine Peptidase 1	Regulation of cell growth	5
IL17RD	Interleukin 17 Receptor D	Regulation of cell growth	—
ITGB4	Integrin, Beta 4	Cell adhesion	—
MGP <sup>a</sup>	Matrix Gla Protein	Regulation of cell growth	—
NID2 <sup>a</sup>	Nidogen 2 (Osteonidogen)	Cell adhesion; basement membrane organization	—
NID2 <sup>a</sup>	Nidogen 2 (Osteonidogen)	Cell adhesion; basement membrane organization	—
NID2 <sup>a</sup>	Nidogen 2 (Osteonidogen)	Cell adhesion; basement membrane organization	—
NID2 <sup>a</sup>	Nidogen 2 (Osteonidogen)	Cell adhesion; basement membrane organization	—
NRG2	Neuregulin 2	Cell growth and differentiation	—
PAQR7	Progestin And AdipoQ Receptor Family Member VII	Protein kinase activity	—
PLAU	Plasminogen Activator, Urokinase	Degradation of extracellular matrix	—
PRKCD <sup>a</sup>	Protein Kinase C, Delta	Regulation of cell growth and apoptosis	—
SH3D19 <sup>a</sup>	SH3 Domain Containing 19	Cell morphology and cytoskeleton organization	—
TMEM35 <sup>a</sup>	Transmembrane Protein 35	Regulation of cell growth	—
TP53BP2 <sup>a</sup>	Tumor Protein P53 Binding Protein, 2	Apoptosis and cell growth regulation	1
TSKU <sup>a</sup>	Tsukushi, Small Leucine Rich Proteoglycan	Morphogenesis; gene expression	—
<b>Gene expression</b>			
BHLHE40 <sup>a</sup>	Basic Helix-Loop-Helix Family, Member E40	Transcriptional regulation	—
CTBP1 <sup>a</sup>	C-Terminal Binding Protein 1	Transcriptional repression	—
ETV4 <sup>a</sup>	Ets Variant 4	Transcriptional regulation	—
GTF2B	General Transcription Factor IIB	Initiation of transcription	—
HOPX <sup>a</sup>	HOP Homeobox	Transcriptional regulation	1
LDB1 <sup>a</sup>	LIM Domain Binding 1	Transcriptional regulation	—
MSX2 <sup>a</sup>	Msh Homeobox 2	Transcriptional repression	—
PIR <sup>a</sup>	Pirin (Iron-Binding Nuclear Protein)	Transcriptional regulation	—
SRPK1 <sup>a</sup>	SRSF Protein Kinase 1	Transcriptional regulation	—
<b>Metabolism</b>			
B3GNT2 <sup>a</sup>	UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase 8	Protein biosynthesis and metabolism	—
BCO2 <sup>a</sup>	Beta-Carotene Oxygenase 2	Carotenoid metabolism	—
ENPP1 <sup>a</sup>	Ectonucleotide Pyrophosphatase/Phosphodiesterase 1	Metabolism	—

(continued)

Table 2. Continued

Gene Symbol	Gene Name	Putative Pouch Function	Cluster
<i>HK3</i>	Hexokinase 2	Glucose metabolism	—
<b>Signaling</b>			
<i>DGKA</i> <sup>a</sup>	Diacylglycerol Kinase, Alpha 80 kDa	Signal transduction	—
<i>LRP8</i> <sup>a</sup>	Low Density Lipoprotein Receptor-Related Protein 8, Apolipoprotein E receptor	Signal transduction; proteolysis	—
<i>OPHN1</i> <sup>a</sup>	Oligophrenin 1	Signal transduction	—
<i>PDE4B</i> <sup>a</sup>	Phosphodiesterase 4B, CAMP-Specific	Signal transduction	—
<i>SCN1A</i>	Sodium Channel, Voltage-Gated, Type I, Alpha Subunit	Voltage-gated sodium channel activity	1
<i>SLC6A7</i> <sup>a</sup>	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 7	Sodium-dependent neurotransmitter transport	—
<i>TSC22D2</i> <sup>a</sup>	TSC22 Domain Family, Member 2	Osmosensory signal transduction	—
<b>Immune regulation</b>			
<i>CCL19</i>	Chemokine (C-C Motif) Ligand 19	Inflammation	—
<i>CEBPB</i>	CCAAT/Enhancer Binding Protein (C/EBP), Alpha	Innate immunity and inflammation (transcription factor)	—
<i>CMTM6</i> <sup>a</sup>	CKLF-Like MARVEL Transmembrane Domain Containing 6	Chemotaxis	—
<i>IL20RB</i> <sup>a</sup>	Cytokine receptor family member B16	Inflammation	—
<i>KLF4</i>	Kruppel-Like Factor 4 (Gut)	Inflammation (inflammatory monocyte differentiation)	1
<i>PDCD1LG2</i> <sup>a</sup>	Programmed Cell Death 1 Ligand 2	Immune response	5
<i>PLGRKT</i> <sup>a</sup>	Plasminogen Receptor, C-Terminal Lysine Transmembrane Protein	Inflammation	—
<i>SART3</i>	Squamous Cell Carcinoma Antigen Recognized By T Cells 3	Induction of cytotoxic T lymphocytes	—
<i>TNFSF12</i>	Tumor Necrosis Factor (Ligand) Superfamily, Member 12	Promotion of inflammatory cytokine secretion	1
<i>XPB1</i> <sup>a</sup>	X-Box Binding Protein 1	Transcriptional regulation; immune response	—
<b>Other</b>			
— <sup>a</sup>	ENSGACG0000001288	Uncharacterized protein	—
— <sup>a</sup>	CABZ01102039.1	Uncharacterized protein	—
— <sup>a</sup>	Uncharacterized protein	Uncharacterized protein	7
— <sup>a</sup>	Uncharacterized protein	Uncharacterized protein	—
<i>ASL</i> <sup>a</sup>	Argininosuccinate lyase	Ammonia detoxification	—
<i>BTR30</i> <sup>a</sup>	Bloodthirsty-related gene family, member 30	Metal ion binding	—
<i>FAM20C</i> <sup>a</sup>	Family With Sequence Similarity 20, Member C	Kinase; Ion binding	—
<i>FRRS1</i> <sup>a</sup>	Ferric-Chelate Reductase 1	Metal ion binding	—
<i>GLT8D2</i> <sup>a</sup>	Glycosyltransferase 8 Domain Containing 2	Transferase activity	—
<i>GPET1</i> <sup>a</sup>	G Protein-Coupled Estrogen Receptor 1	Estrogen-dependent; smooth muscle contraction	—
<i>HSD11B2</i> <sup>a</sup>	Hydroxysteroid (11-Beta) Dehydrogenase 2	Steroid binding	—
<i>MAT2A</i> <sup>a</sup>	Methionine Adenosyltransferase II, Alpha	Cellular processes	—
<i>PAPSS1</i>	3'-Phosphoadenosine 5'-Phosphosulfate Synthase 1	Sulfate activation	—
<i>POR</i> <sup>a</sup>	P450 (Cytochrome) Oxidoreductase	Electron transport	—
<i>SLC25A1</i> <sup>a</sup>	Solute Carrier Family 25 (Mitochondrial Carrier; Citrate Transporter), Member 1	Mitochondrial citrate transport	—

NOTE.—Cluster membership refers to the expression clusters shown in figure 3.

<sup>a</sup>Highly downregulated genes (the 30 most significantly downregulated genes in each pairwise comparison).

transporter that is known to increase in concentration in blood plasma during human pregnancy (Ahnstrom et al. 2010). *APOM* and additional upregulated lipid transporters (*APOA1* and *APOA2* [Cluster 4]; *CETP*; *FABP1*, 3, and 7; table 1) may promote the transport of lipids across the brood pouch epithelium to the developing embryos, which have very high lipid requirements (Faleiro and Narciso 2010), a process which may explain the large quantities of lipids that

have been observed in histological studies of pouch epithelial cells (Linton and Soloff 1964).

The pouch fluid of pregnant seahorses is protein-rich (Boisseau 1967), but we identified few differentially regulated amino acid or peptide transporters during pregnancy, making it likely that the protein-rich fluid is largely maternally derived (Boisseau 1967). Excess protein contained in the seahorse egg (55% protein vs. 37% lipids by dry weight; Alvarez et al. 2009)

**Table 3.** Significantly Upregulated Genes in the Postparturition Seahorse Pouch Compared with the Late-Pregnant Pouch.

Gene Symbol	Gene Name	Putative Pouch Function	Cluster
<b>Cell growth, differentiation, and apoptosis</b>			
ABHD2 <sup>a</sup>	Abhydrolase Domain Containing 2	Migration of smooth muscle cells	—
ABL2	C-Abl Oncogene 2, Nonreceptor Tyrosine Kinase	Cytoskeleton remodeling	5
ABL2	C-Abl Oncogene 2, Nonreceptor Tyrosine Kinase	Cytoskeleton remodeling	5
ABL2	C-Abl Oncogene 2, Nonreceptor Tyrosine Kinase	Cytoskeleton remodeling	5
ACAN <sup>a</sup>	Aggrecan	Extracellular matrix involvement	—
CASP3	Caspase 3, Apoptosis-Related Cysteine Peptidase	Apoptosis	7
CEACAM1 <sup>a</sup>	Carcinoembryonic Antigen-Related Cell Adhesion Molecule 1 (Biliary Glycoprotein)	Cell adhesion; apoptosis; immunity	—
CYCS	Cytochrome C, Somatic	Apoptosis	—
DDIT4	DNA-Damage-Inducible Transcript 4	Apoptosis	—
DUSP5 <sup>a</sup>	Dual Specificity Phosphatase 5	Kinase inactivation	—
EGFR	Epidermal Growth Factor Receptor	Cell proliferation	—
HSPB11 <sup>a</sup>	Heat Shock Protein Family B (Small), Member 11	Cell adhesion	5
HTRA1	HtrA Serine Peptidase 1	Regulation of cell growth	5
HTRA1	HtrA Serine Peptidase 1	Regulation of cell growth	5
LARP4 <sup>a</sup>	La Ribonucleoprotein Domain Family, Member 4	Cytoskeleton organization	5
MEF2A	Myocyte Enhancer Factor 2A	Cell growth and survival	7
MEF2C	Myocyte Enhancer Factor 2C	Myogenesis	—
MINK1	Misshapen-Like Kinase 1	Cytoskeleton remodeling, cell migration	—
OXSR1	Oxidative Stress Responsive 1	Cytoskeleton regulation	—
PAK4	P21 Protein (Cdc42/Rac)-Activated Kinase 4	Cytoskeleton regulation	6
ROCK2	Rho-Associated, Coiled-Coil Containing Protein Kinase 2	Cytoskeleton regulation, focal adhesion	—
si:dkeyp-75b4.10 <sup>a</sup>	Novel protein containing a C-type lectin domain	Growth factor activity	—
STK4	Serine/Threonine Kinase 3	Apoptosis; growth repression	—
TGFA	Transforming Growth Factor, Alpha	Cell proliferation	—
TIMM50	Translocase of Inner Mitochondrial Membrane 50 Homolog ( <i>S. cerevisiae</i> )	Apoptosis	—
zgc:100868 <sup>a</sup>	zgc:100868	Proteolysis	—
<b>Gene expression</b>			
ATF3	Activating Transcription Factor 3	Regulation of transcription	—
CCRN4L <sup>a</sup>	CCR4 Carbon Catabolite Repression 4-Like ( <i>S. cerevisiae</i> )	Regulation of transcription	5
EPAS1	Endothelial PAS Domain Protein 1	Regulation of transcription	—
HMGA1	High Mobility Group AT-Hook 2	Regulation of transcription	—
RCAN1 <sup>a</sup>	Regulator Of Calcineurin 1	Regulation of transcription	5
SRF	Serum Response Factor (C-Fos Serum Response Element-Binding Transcription Factor)	Transcriptional regulation; cellular proliferation	5
SRPK1 <sup>a</sup>	SRSF Protein Kinase 1	RNA splicing	—
TSC22D2 <sup>a</sup>	TSC22 Domain Family, Member 2	Regulation of transcription	—
XBP1	X-Box Binding Protein 1	Regulation of transcription	—
<b>Signaling</b>			
ARF5 <sup>a</sup>	ADP-Ribosylation Factor 5	Signal transduction	5
FAM13B <sup>a</sup>	Family With Sequence Similarity 13, Member B	Signal transduction	—
FAM13B	Family With Sequence Similarity 13, Member B	Signal transduction	7
PDE4B <sup>a</sup>	Phosphodiesterase 4B, CAMP-Specific	Signal transduction	—
SLC6A7 <sup>a</sup>	Solute Carrier Family 6 (Neurotransmitter Transporter), Member 7	Sodium-dependent neurotransmitter transport	—
STOML3 <sup>a</sup>	Stomatin (EPB72)-Like 3	Signal transduction	—
<b>Transport</b>			
SLC11A2	Solute Carrier Family 11 (Proton-Coupled Divalent Metal Ion Transporter), Member 2	Metal ion transport	5
SLC16A6	Solute Carrier Family 16, Member 6	Monocarboxylate transport	—
SLC22A4	Solute Carrier Family 22 (Organic Cation/Zwitterion Transporter), Member 4	Cation/sodium cotransport	—

(continued)

Table 3. Continued

Gene Symbol	Gene Name	Putative Pouch Function	Cluster
SLC23A2	Solute Carrier Family 23 (Ascorbic Acid Transporter), Member 1	Sodium/ascorbic acid cotransport	—
SLC2A3	Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 3	Glucose transport	—
SLC2A3	Solute Carrier Family 2 (Facilitated Glucose Transporter), Member 3	Glucose transport	—
SLC47A2	Solute Carrier Family 47 (Multidrug and Toxin Extrusion), Member 2	Toxin transport	5
SLC5A3	Solute Carrier Family 5 (Sodium/Myo-Inositol Cotransporter), Member 3	Myo-inositol transport	—
SLC9A1	Solute Carrier Family 9, Subfamily A (NHE1, Cation Proton Antiporter 1), Member 1	Na <sup>+</sup> /H <sup>+</sup> transport; pH regulation	6
SLCO2A1	Solute Carrier Organic Anion Transporter Family, Member 2A1	Prostaglandin transport	—
YKT6 <sup>a</sup>	YKT6 V-SNARE Homolog ( <i>S. cerevisiae</i> )	Endoplasmic reticulum transport	—
Immune regulation			
IL20RB <sup>a</sup>	Cytokine receptor family member B16	Inflammation	—
PDCD1LG2 <sup>a</sup>	Programmed Cell Death 1 Ligand 2	Immune response	5
Other			
BCO2 <sup>a</sup>	Beta-Carotene Oxygenase 2	Carotenoid metabolism	5
FASN <sup>a</sup>	Fatty Acid Synthase	Fatty acid synthesis	5
FRRS1 <sup>a</sup>	Ferric-Chelate Reductase 1	Metal ion binding	—
GPBR1	G Protein-Coupled Estrogen Receptor 1	Estrogen-dependent; smooth muscle contraction	—
IST1 <sup>a</sup>	Increased Sodium Tolerance 1 Homolog (Yeast)	Protein localization	—
MAP2K2	Mitogen-activated protein kinase 2	Parturition	6
MAPK9	Mitogen-activated protein kinase 9	Parturition	5
MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2	Parturition	6
MAT2A <sup>a</sup>	Methionine Adenosyltransferase II, Alpha	Metabolism	—
TMEM35 <sup>a</sup>	Transmembrane Protein 35	Neurite growth regulation	—
zgc:110353 <sup>a</sup>	Uncharacterized protein		7
zgc:123010 <sup>a</sup>	zgc:123010	Nucleic acid binding	5

NOTE.—Cluster membership refers to the expression clusters shown in figure 3.

<sup>a</sup>Highly upregulated genes (the 30 most significantly upregulated).

may diffuse across the chorion and contribute to the pouch fluid, before being lysed by pouch proteases (Boisseau 1967). Consistent with this hypothesis, we detected the upregulation of proteases in the pregnant pouch (e.g., MMP19; TMPRSS2 [Cluster 3]; PRSS59.1). These proteases may originate from the modified flame cone cells lining the *Hippocampus* brood pouch epithelium (Bereiter-Hahn et al. 1980; reviewed in Carcupino et al. 2002; Laksanawimol et al. 2006). Further studies using in situ hybridization to localize protease genes to particular cell types are required to confirm this hypothesis.

Our gene expression data suggest that seahorse patrotrophy may involve the transfer of inorganic ions to embryos. Calcium and iron, both of which are found in pregnant seahorse brood pouch fluid, are absorbed by developing seahorse embryos (Linton and Soloff 1964; Oconer et al. 2006). We detected the upregulation of transporter genes for Ca<sup>2+</sup>, K<sup>+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup>, Mg<sup>2+</sup>, and Fe<sup>2/3+</sup> in the pregnant seahorse brood pouch (ATP2C1 [Cluster 9], KCNK5 [Cluster 3], SLC30A7, SLC31A2, SLC39A6, SLC41A1, FTH1 and TF). During mammalian pregnancy, TF captures iron ions before binding Transferrin Receptor (TFR) at the placental epithelium, promoting iron transport to the embryo (Gambling et al. 2011). Given that TFR is also expressed in the seahorse brood pouch (supplementary table S13, Supplementary Material online), we hypothesize that the TFR/TF pathway may play a role in iron transport from father to embryos. The majority of the

upregulated inorganic ion transporters reaches peak expression at mid-pregnancy (supplementary table S12, Supplementary Material online), when embryonic demand for inorganic ions is highest (Oconer et al. 2006). In particular, calcium binding and transport by genes such as ATP2C1 (Cluster 9) may be crucial to meet the high calcium demand of embryonic skeletal and bony body ring development.

#### Transport II: Ion Transporters and Osmoregulation

The pouch fluid of *Hippocampus* sp. falls to paternal serum osmolarity early in pregnancy before gradually increasing to seawater osmolarity prior to parturition, which serves to acclimate embryos to the external environment before release (Leiner 1934; Linton and Soloff 1964), a pattern also found in some pipefish (Ripley 2009). Influx of seawater into the pouch during early development damages embryos (Fiedler 1954), suggesting that stable osmotic conditions may be necessary to prevent embryonic deformity or physiological defects (reviewed in Quast and Howe 1980). Although sodium is transported from the pouch fluid to the blood of early pregnant seahorses and there is no direct exchange from pouch to the surrounding seawater during early pregnancy (Linton and Soloff 1964), the mechanisms underlying osmoregulation in this system remain unknown (Linton and Soloff 1964; Carcupino et al. 2002).



The stable expression of  $\text{Na}^+/\text{K}^+$ -transporting ATPase genes observed in both pregnant and nonpregnant animals (supplementary table S13, Supplementary Material online) suggests that the seahorse brood pouch maintains its osmoregulatory function regardless of reproductive status. Additional ion transport during early pregnancy may be facilitated by two *SLC4A10* transcripts, which are putative sodium/bicarbonate cotransporters (Romero et al. 2004) and reach peak expression during early pregnancy (Cluster 2). We hypothesize that sodium/bicarbonate exchange in the seahorse brood pouch may function in a similar manner to fish osmoregulation across the intestinal epithelium (Cutler and Cramb 2001). In contrast to the pronounced upregulation of putative sodium/bicarbonate cotransporters during early pregnancy, we did not identify any ion transporters showing differential expression during late pregnancy, when pouch osmolality starts to increase to match the external environment (Linton and Soloff 1964; Oconer et al. 2006). We suggest that osmolarity increases prior to parturition may be produced by the controlled entry of seawater into the pouch, analogous to the uterine flushing of some viviparous chondrichthyan fishes (Evans et al. 1982). Pouch flushing during late pregnancy would also serve to meet the growing oxygen and waste removal demands of late stage embryos.

### Transport III: Solute and Waste Removal

Although the pregnant brood pouch requires the capacity to remove and/or store waste materials during embryonic development, this function has not yet been experimentally demonstrated. Our expression analysis identified four upregulated transcripts encoding putative  $\text{CO}_2$  transporters (*CA2*, *CA4*, *RHAG*, and *SLC4A1* [Cluster 4]), providing candidates for future experimental work exploring the physiology of gas exchange between embryonic and paternal tissues. These genes are known to maintain bicarbonate balance in humans (Romero et al. 2004), and concurrent increases in the activity of genes such as the  $\text{H}^+$  transporter *ATP5L2* may help to buffer pouch fluid pH.

Nitrogen accumulates in seahorse brood pouch fluid during pregnancy, a process thought to be the byproduct of the secretion of ammonia by developing embryos, before decreasing to zero by parturition (Oconer et al. 2006). Although ammonia is inherently highly diffusible, the closed brood pouch environment limits passive diffusion. *RHAG*, a  $\text{CO}_2$  transporter, plays an important role in ammonia excretion from the fish gill (Nakada et al. 2007), and is upregulated from late pregnancy until after parturition (supplementary table S12, Supplementary Material online); pouch *RHAG* expression may enable the active transport of ammonia as well as  $\text{CO}_2$  out of the brood pouch, contributing to nitrogen decreases in brood pouch fluid before parturition (Oconer et al. 2006). Additionally, although the majority of marine fish are predominantly ammonotelic, seahorses excrete urea (Wilson et al. 2006), and the upregulation of a gene encoding a putative argininosuccinate lyase (*ASL*), which detoxifies ammonia through the urea cycle, may play a role in this process

during mid-late pregnancy (supplementary table S12, Supplementary Material online).

### Immune Regulation

Both innate and adaptive immune genes are differentially regulated in the seahorse brood pouch during pregnancy. The seahorse brood pouch, open to the external environment in the nonreproductive state (Whittington et al. 2013), is nonsterile, and following egg transfer, embryos are brooded in an enclosed, nutrient-rich environment that is ideal for bacterial growth. Interestingly, one of the most highly upregulated transcripts at all stages of pregnancy is *LECT2* (supplementary table S12, Supplementary Material online), encoding a neutrophil chemotaxis protein associated with bacterial resistance in fish (Fu et al. 2014). Other upregulated innate immune genes include hepcidin (*HAMP*; Cluster 4), which encodes an antifungal peptide (Park et al. 2001), and a toll-like receptor gene (*TLR18*; Cluster 9), which functions in pathogen recognition. We also recovered highly (but not differentially) expressed C-type lectin genes (*CLEC*; supplementary table S13, Supplementary Material online), which may protect seahorse and pipefish embryos from microbial infection (Melamed et al. 2005; Small et al. 2013).

The traditional mammalian view of a fetus as an allograft (Moffett and Loke 2006), in which the maternal immune system must be tightly regulated to prevent embryonic rejection (Walker et al. 2010), may also apply to seahorse embryos, which are in direct contact with the paternal pouch epithelium during gestation. Immune transcripts downregulated during seahorse pregnancy include genes associated with inflammation (e.g., *IL20RB*, *PLGRKT*) (table 2); a reduction in the inflammatory response during pregnancy may prevent activation of the adaptive immune system (Challis et al. 2009). Interestingly, although adaptive immune genes are often differentially regulated during mammalian pregnancy to promote fetal tolerance (Davies et al. 2000), we found that several genes associated with allorecognition in mammals are not differentially regulated during seahorse pregnancy (e.g., *MHC1UBA*, *MHC1ZBA*, *TRBC2*, and *ZAP70*; supplementary table S13, Supplementary Material online). These results may reflect differences in the generalized immune response in fish and mammals, or may indicate that allorecognition is less important in the pregnant seahorse, where embryos contact, but do not invade into, paternal tissues (Oconer and Herrera 2003).

### Other Highly Differentially Expressed Genes

An astacin-like metalloprotease (*ASTL*) is highly upregulated during mid- and late-pregnancy (supplementary table S12, Supplementary Material online) and exhibits sequence similarities to both a recently described brood pouch “patristacin” of unknown function in the pipefish (Harlin-Cognato et al. 2006; Small et al. 2013), and the high choriolytic enzyme (*HCE*/choriolytin H) of oviparous fishes (Inohaya et al. 1995). HCEs are expressed in embryonic hatching glands (Inohaya et al. 1995) and act to lyse the chorion that surrounds the egg, allowing the embryo to hatch into the external environment (Yasumasu et al. 1989). Seahorse embryos hatch from their chorion at mid- to late pregnancy, but are

retained in the pouch (Wetzel and Wourms 2004; Sommer et al. 2012). Given the pattern of seahorse *ASTL* gene expression across pregnancy and the degree of sequence similarity to HCEs, we speculate that this gene may be involved in regulating the timing of embryonic hatching within the brood pouch.

Another highly upregulated gene across all stages of pregnancy, particularly early-mid pregnancy, is a member of the mucin family, oviductal glycoprotein 1 (*OVGP1*) (Cluster 8), whose expression correlates with increases in glycoprotein in the pregnant pouch (Linton and Soloff 1964). In mammals, *OVGP1* is secreted from the oviduct epithelium under estrogen control, particularly during gamete transport and early embryogenesis (Verhage et al. 1988).

### Parturition

Our unique experimental design, incorporating multiple reproductive time points, allows us to identify genes associated with seahorse parturition, offering an intriguing glimpse into a poorly understood process. Postparturition is associated with the highest number of differentially expressed transcripts of any reproductive stage tested (753 transcripts differentially expressed compared with late pregnancy; [fig. 2](#)), and is dominated by the upregulation of genes associated with cell growth and differentiation, cytoskeleton remodeling, and apoptosis ([table 3](#)), suggesting that tissue repair, and remodeling are important functions in the postreproductive brood pouch. The rapid remodeling of the brood pouch after pregnancy may facilitate the short interbrood intervals found in this group (Foster and Vincent 2004; Bahr et al. 2012). Interestingly, all of the most significantly downregulated genes after parturition ([supplementary table S12, Supplementary Material online](#)) are upregulated in early-, mid-, and/or late-pregnant animals relative to both nonpregnant and postparturition individuals, suggesting that pouch functions may start to revert to the nonpregnant state soon after parturition.

The endocrinological regulation of syngnathid reproduction remains poorly understood (Scobell and MacKenzie 2011; Whittington and Wilson 2013), and our study offers an opportunity to investigate the hormonal control of parturition. KEGG pathway analysis identified the postparturition upregulation of genes associated with the MAPK/ERK signaling pathway ([supplementary table S11, Supplementary Material online](#)), which is activated by estrogen in mammals (Prossnitz and Maggiolini 2009). MAPKs are activated by uterine stretching in pregnant mammals and produce signals associated with the onset of labor (Oldenhof et al. 2002). As estradiol induces premature parturition when administered during the first half of seahorse pregnancy (Boisseau 1965, 1967) and the male brood pouch is significantly distended during late pregnancy, we suggest that estrogen signaling and mechanical stretching of the pouch by the developing embryos may stimulate parturition in pregnant seahorses. This hypothesis could be tested by measuring the expression of genes associated with the MAPK/ERK pathway in male seahorses before and after the experimental administration of estradiol.

Notably, we detected the postparturition upregulation of the estrogen receptor *GPER1* ([table 3](#) and [supplementary table S12, Supplementary Material online](#)), which mediates increased uterine myometrial contractility in mammals in response to oxytocin (Maiti et al. 2011; Tica et al. 2011). In seahorses, isotocin (the fish ortholog of oxytocin) induces characteristic parturition-like pouch compression movements when injected into the brood pouch of nonpregnant males (Fiedler 1970), a process which may be mediated by *GPER1*. Pouch compressions are also an important component of seahorse courtship (Whittington et al. 2013), which may explain the presence of *GPER1* expression in nonpregnant seahorses ([supplementary table S12, Supplementary Material online](#)). We hypothesize that *GPER1* is downregulated during pregnancy to ensure pouch quiescence, and then upregulated during parturition in response to isotocin to produce the smooth muscle contractions facilitating offspring release.

### Common Gene Use in Vertebrate Pregnancy and Parturition

Syngnathid pregnancy represents the independent origin of a unique form of viviparity in which embryos are brooded by males in a complex brooding organ that is derived from a distinctive tissue type. As such, the range of genes showing consistent expression profiles in both male seahorses and viviparous female vertebrates during pregnancy is particularly striking, and warrants further discussion.

Recent research suggests that independent origins of viviparity in amniotes may have evolved using similar genetic pathways (Murphy and Thompson 2011; Brandley et al. 2012; Van Dyke et al. 2014). Our study extends these comparisons to anamniotes, where we have found that many genes in the reproductive transcriptome of pregnant male seahorses are homologous to those found in the uterus of female mammals and squamate reptiles. In addition to the genes discussed in detail above (e.g., *MAPK/ERK*, *ASTL*, *OVGP1*), Caveolin 1 (*CAV1*) and protein phosphatase 1, regulatory subunit 12A (*PPP1R12A*) are both upregulated in pregnant seahorse brood pouch ([supplementary table S12, Supplementary Material online](#)) as well as during rat pregnancy (Lontay et al. 2010; Madawala et al. 2011). Similarly, we find that *MMP19* (a bovine endometrial remodeling gene; Bauersachs et al. 2008), and *PHLDA1* and *DLX3* (both regulators of mammalian placental growth; Morasso et al. 1999; Salas et al. 2004) are all upregulated during seahorse pregnancy ([supplementary table S12, Supplementary Material online](#)). Remarkably, genes expressed during gestation in the platyfish ovary that are associated with placental function in mammals are also detected in the seahorse brood pouch, including *PPARG* and *NCOA6* ([supplementary table S13, Supplementary Material online](#)) (Schartl et al. 2013). Other differentially expressed seahorse brood pouch transcripts are homologous to additional viviparous teleost placental genes, including the protease inhibitor/innate immune gene *A2M* (Panhuis et al. 2011) ([supplementary table S12, Supplementary Material online](#)). Finally, pregnancy-associated lipid transporters in mammals and lizards (e.g., *FABPs* [Duttaroy 2009; Brandley et al. 2012], apolipoproteins

[Brandley et al. 2012], and *CETP* [Iglesias et al. 1994]) are also significantly upregulated during seahorse pregnancy (see above), suggesting that similar genetic pathways may be associated with nutrient transport in the amniote uterus and the seahorse brood pouch.

There are several explanations for the shared gene use that we observe across independent origins of vertebrate pregnancy. First, transcripts associated with pregnancy in seahorses, other fish, mammals, and reptiles may be derived from genes with broad tissue expression domains in ancestral taxa including both the abdominal epidermis (seahorse; Carcupino et al. 2002) and the female reproductive tract (mammals/reptiles/other fish). Under this scenario, shared gene use during pregnancy would result from the shared evolutionary history of ancestral cell types in a common vertebrate ancestor (Arendt 2008), followed by exaptation to the common challenges of gestation, rather than the convergent recruitment of expression of the same genes to de novo organ systems. Alternatively, these same suites of genes may have been repeatedly and independently recruited (e.g., True and Carroll 2002; Knox and Baker 2008) into expression in the mammalian uterus, the reptilian uterus, the poeciliid ovary, and the seahorse brood pouch as these organ systems increased in complexity during their evolution. A recent study indicates that large numbers of genes evolved endometrial expression coincident with the origin of mammalian pregnancy (Lynch et al. 2015), and similar processes may underlie the origin of other forms of viviparity. Future studies should test these alternatives by examining gene expression changes in viviparous species and their oviparous relatives to determine how patterns of gene expression have changed during the evolution of live bearing, and whether similar mechanisms have been used in independent origins of viviparity.

The complex challenges of ensuring gas exchange, waste removal, nutrient provisioning, and immunological protection during internal incubation likely constrain the evolution of viviparity, and the use of many of the same genes in the reproductive transcriptomes of male seahorses, female viviparous fish, and viviparous amniotes suggests that these animals may have overcome the challenges of pregnancy in similar ways. The use of many of the same genes in reproductive processes across diverse taxa, regardless of the sex or the tissue type used to incubate embryos, implies that the evolution of the reproductive transcriptome may be nonrandom and, to some extent, predictable (Stern 2013). It is important to note that differences in pregnancy genes between viviparous taxa are also informative, as there may be multiple genetic means to achieve the same phenotypic end (e.g., Manceau et al. 2010; Elmer and Meyer 2011; Losos 2011; Whittington et al. 2015). Although this is a particularly exciting area for future research, the large numbers of unannotated gene products identified in de novo transcriptomes (here approximately 36% of differentially regulated transcripts) will likely pose a formidable challenge to studies aiming to identify unique morphological and physiological solutions to internal reproduction in nonmodel organisms. Further comparative studies of closely related viviparous

species showing key differences in gestational physiology (e.g., different mechanisms of osmoregulation, immunological protection, and/or nutrient transport) may be helpful to identify promising candidate genes for functional characterization. Finally, if transitions to viviparity across vertebrates are dominated by consistent genetic changes across broad classes of genes, this may indicate the presence of genetic constraints governing the evolution of complex forms of reproduction. Further comparative studies of a broad sampling of viviparous taxa, including amphibians, chondrichthyans, and viviparous invertebrates, will be needed to investigate this possibility.

## Materials and Methods

### Animals

Reproductively mature male and female pot-bellied seahorses (*H. abdominalis*) derived from a captive-bred population (Seahorse Australia, Tasmania, Australia) were maintained under standard laboratory conditions as previously described (Whittington et al. 2013); CITES approval number 2011-AU-646379/PWS P105545. Animals were allowed to mate freely before being subjected to a standardized assessment of pregnancy status on the basis of courtship behaviors (Whittington et al. 2013). Pregnant seahorses were maintained in single-sex tanks before euthanasia to sample pouch tissues at key stages throughout pregnancy. The targeted time periods were: 1–3 days postfertilization (dpf) (early), 7–10 dpf (mid), 14–17 dpf (late), postparturition (< 12 h after embryo release), and non-pregnant (fig. 1). We sampled three animals per time point (i.e., three biological replicates). Staging of embryos revealed that pregnancy stages correspond to the early embryogenesis (early), optic cup/eye pigmentation (mid), frontal-jaw (late), and release (postparturition) stages of the standardized syngnathid embryo classification system (Sommer et al. 2012).

### Tissue Sampling

Animals of known reproductive stage were transferred to a 10-l container of seawater from the main system, with an artificial seagrass holdfast to allow attachment. After 10 min, animals were euthanized by decapitation according to approved protocols (Veterinär Zürich Permit 164/2010). The pouch and abdominal cavity were opened using surgical scissors and the whole animal was submerged in RNAlater (Sigma-Aldrich, St Louis, MO) before being stored at 4 °C overnight. Following the removal of embryos, the pouch was gently washed with deionized water to remove embryonic contamination. The inner lining of the pouch was then carefully excised and stored at –80 °C until RNA extraction. Total RNA was extracted in randomized batches using an RNeasy Mini Kit (Qiagen, Hilden Germany) with QiaShredder (Qiagen) and on-column DNase digestion (RNase-free DNase set, Qiagen).

### Sequencing, Assembly, and Annotation

RNA quality was assessed using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). After library preparation, samples were sequenced on 2.5 lanes of an Illumina HiSeq (Illumina, San Diego, CA) with 100-bp paired-end (PE)



reads. The resulting 723.6 million raw PE reads were quality filtered and trimmed of adaptor contamination and assembled into a reference transcriptome de novo using Trinity (Haas et al. 2013). Adaptor trimming was done using FLEXBAR version 2.3 (Dodt et al. 2012). Quality trimming at both ends (Q20) and filtering (minimum mean quality of Q30, minimum read length of 25 bp) were performed with PRINSEQ-lite version 0.17.4 (Schmieder and Edwards 2011). To remove contaminant reads introduced during sequencing, sequences were also mapped to the contaminant genome using Bowtie 2 version 2.0.6 (Langmead and Salzberg 2012) and only unmapped reads were retained for the downstream assembly. Trinity assembly of trimmed and filtered reads was performed using default parameters except for the `min_kmer_cov` variable, which was set to 2, resulting in 259,151 contigs with an N50 of 2,544 bp and median length of 574 bp. We treated the output of Trinity clusters as sets of genes that share sequence, and collapsed isoforms allocated to the same gene identifier.

The resulting sequences were annotated by aligning contigs to the Ensembl (Ensembl Genes 71) *Danio rerio* (Zv9) and *Gasterosteus aculeatus* (BROADS1) proteomes using NCBI BLASTX (version 2.2.28+) with default parameters. Contigs were annotated as the gene with the highest similarity with an expectation value less than  $10^{-5}$ . Due to the different systems of nomenclature used for each genome, downstream analyses were based on the *D. rerio* annotation, which more closely approximates the standard Human Genome Organisation nomenclature system, facilitating downstream analyses. In instances in which significant similarity to *D. rerio* was not detected, the *G. aculeatus* annotation was used. This approach resulted in the annotation of 35.2% of seahorse transcripts (an improvement of 1.5% over *D. rerio* alone). Annotated transcripts included 16,688 unique Ensembl IDs.

Differentially expressed genes were identified in two-group analysis of each of the ten possible comparisons of the five stages of pregnancy, which was executed using the exactTest method implemented in the Bioconductor package edgeR (Robinson et al. 2010). Sequences that were significantly up- or downregulated in any one of the two-group comparisons (Benjamini-adjusted  $P < 0.05$ ) were included in our downstream analysis. Abundance estimates of assembled transcripts were obtained using RSEM version 1.2.0, which computes maximum-likelihood abundance estimates using the Expectation–Maximization algorithm (Li and Dewey 2011). Briefly, RSEM mapped PE reads back to the reference transcriptome assembled by Trinity using bowtie (Langmead et al. 2009). Before read alignment, read ends were both hard-trimmed (5 bases) and quality trimmed (Q20) using PRINSEQ-lite version 0.17.4. Transcripts per million values (Li and Dewey 2011) were used as the measure of abundance and were normalized using the log mean method (Robinson et al. 2010). Genes with expression abundances below 10 were excluded from this analysis. Pairwise comparisons of expression differences between sequential reproductive stages identified a minimum of 18 differentially expressed transcripts (between mid- and late pregnancy) and a maximum of 753 differentially expressed transcripts (between late pregnancy

and postparturition) (fig. 2). Pairwise comparisons of all reproductive stages identified a total of 3,158 transcripts with significant expression differences between two or more stages of pregnancy.

### Cluster Analysis

Soft clustering was performed on the mean expression values of all unique differentially expressed genes using the mFuzz algorithm, which groups genes with congruent temporal expression profiles into discrete clusters (Futschik and Carlisle 2005). Expression values were normalized to z-scores (mean = 0; SD = 1), and a fuzziness value ( $m = 2.021468$ ) was estimated using the mestimate function, to avoid clustering of random data. Genes with a membership value greater than 0.7 were assigned to each cluster. Genes were clustered into nine expression profiles, which maximized the number of clusters with unique gene expression patterns without producing duplicate clusters with similar expression patterns.

### GO, Functional Grouping, and Pathway Analysis

GO annotations were carried out using DAVID functional annotation tool version 6.7 (available: <http://david.abcc.ncifcrf.gov/home.jsp>, last accessed May 2015) (Huang et al. 2009), with GO\_FAT biological process terms, using all genes expressed in the nonpregnant seahorse brood pouch as background. The Functional Annotation Clustering option (referred to throughout the text as functional annotation grouping in order to differentiate it from the cluster analysis) was used to group significantly enriched GO terms as assessed by a modified Fisher's Exact Test by function using the DAVID Fuzzy clustering algorithm (Huang et al. 2009). Grouping was performed using DAVID settings for medium stringency and  $P$  values were Benjamini-corrected to account for multiple hypothesis testing. GO annotation results are presented for the best annotation Ensembl gene IDs for the following comparisons: Pregnant (early-, mid-, and late-pregnant) versus nonpregnant, and late-pregnant versus postparturition. KEGG pathway analysis with an EASE of 0.1 and Benjamini–Hochberg corrected  $P$  values was also carried out using DAVID.

GO annotation, searches for enriched GO categories, and KEGG pathway analysis were simultaneously carried out using WebGestalt version 1/30/2013 (available: <http://bioinfo.van.derbilt.edu/webgestalt/>, last accessed May 2015) (Wang et al. 2013), with GO\_Slim biological process terms and the *D. rerio* genome as background. This analysis used hypergeometric statistical testing ( $P > 0.05$ ), with Benjamini–Hochberg correction for multiple tests. The results of these analyses are provided as [supplementary tables S7–S10, Supplementary Material online](#).

### Data Access

All sequence data have been uploaded to NCBI's GenBank Sequence Read Archive under accession number SRP049289.



## Supplementary Material

Supplementary tables S1–S13 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

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